In Vitro Innovations

The development of alternative methods for testing chemicals is on the rise. Concerns about the well-known Draize eye and skin tests in which substances are applied to live animals, for example, have centered on animal pain and distress, reproducibility of the data, and subjective evaluations by animal testers. Two new fields of research are aimed at developing innovative skin and eye analogs for *in vitro* testing of chemicals that may eventually replace traditional animal testing.

Skin Deep

The most accessible organ of the human body, skin serves a variety of immunologic, metabolic, sensory, and protective functions while interfacing with the external environment and internal physiology. A variety of human skin models have shown promise in their ability to provide *in vitro* alternatives to animal testing. In addition to their usefulness in toxicological and pharmacological testing, these models are also shedding light on mechanisms of wound healing and the pathophysiology of skin diseases, as well as regulation of skin cell differentiation.

The epidermis, the outermost layer of the skin, consist primarily of keratinocytes, cells that produce the tough protein, keratin. The epidermis produces the stratum corneum or horny cell layer, which serves as a protective, semipermeable membrane surrounding the body. Beneath the epidermis is the dermis, which consists of a tough fibrous network of collagen and elastic fibers that create channels for the flow of nutrients and exchange of metabolites between these two layers of skin.

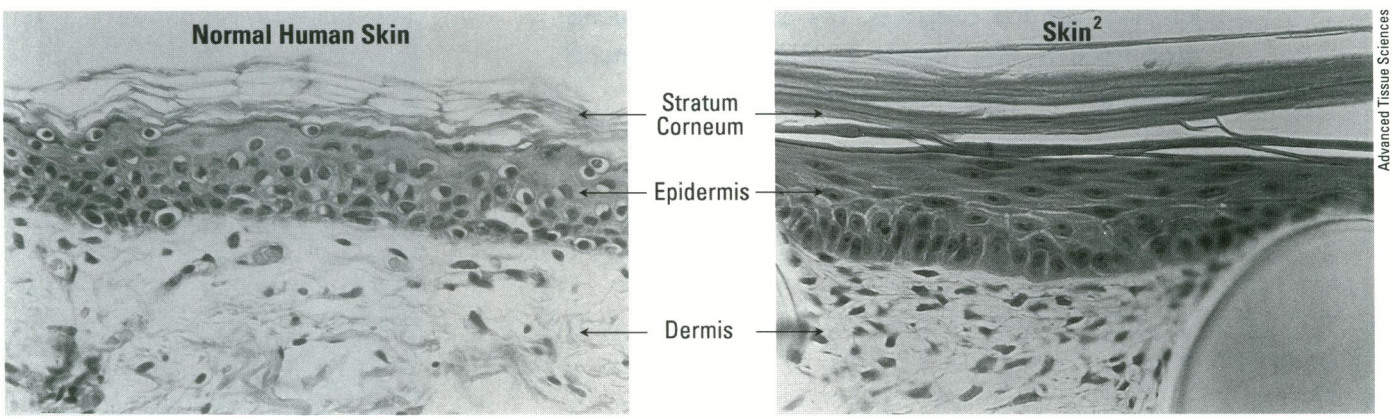
In vitro models of human skin should approximate, as closely as possible, their trilayered, multifunctional, living tissue counterpart. Traditional monolayer cultures of keratinocytes, in which cells are grown while submerged in culture media, show patterns of cell differentiation that are different from intact human skin. Moreover, such models are generally limited to tests involving water-soluble substances, thereby eliminating from *in vitro* testing many of the chemicals (e.g. solids, gels, crystals) with which the body comes into contact.

More recent technologies distribute, or seed, cells onto a support system, or matrix, producing a multilayered epidermis that more closely approximates its *in vivo* counterpart. These three-dimensional skin analogs allow the direct application of a variety of soluble and insoluble materials. Maria Ponec and her colleagues at Leiden University Hospital in the Netherlands designed a three-dimensional epidermis by seeding human keratinocytes onto a biological support called de-epidermized dermis (DED). Ponec's analog is called epidermis reconstructed on DED (RE-DED). The keratinocytes in the RE-DED show a high degree of cell differentiation.

The source of this enhanced differentiation appears to lie in the DED itself. Derived from human cadaver skin, the DED consists of dead skin cells from which the epidermis has been removed. The remaining dermis retains a huge part of the basement membrane that normally forms a tight connection between the epidermis and the dermis. "That's quite an advantage of our system, because when you seed the keratinocytes on top of the DED they adhere much better, which very possibly provides a trigger for differentiation," Ponec explains.

Skin^2, developed by Advanced Tissue Sciences in La Jolla, California, is a three-dimensional skin analog made of cells derived from human foreskins seeded onto a nylon mesh support. "The mesh reproduces the microenvironment that the dermal fibroblasts and normal keratinocytes require. They grow in such a way that they are reminiscent of the environment in situ, in the human body," explains Lawrence Rheins, executive director of the Skin^2 division.

The simplest of these analogs consists of one type of cell, such as the keratinocyte, which forms the epidermis of RE-DED. Early models of Skin^2 consisted of fibroblasts that secrete collagen, growth factors, and proteins that form the extracellular matrix of the dermis. Single-cell models have "an advantage, in that . . . there is no interaction between these cells and other cells," says Ponec. Her studies



Skin deep. *In vitro* models of human skin allow scientists to test chemicals in a petri dish rather than on live animals.

also show that the small protein molecules known as cytokines mediate interactions between mixed cultures of keratinocytes and fibroblasts.

More complex models combine keratinocytes with fibroblasts to produce a dermis and epidermis with varying degrees of differentiation. Which model yields the best information? "That depends on the question you are asking," explains Ponec. Rheins concurs, citing some of the different uses for Skin^2. "The ZK1000 [dermis] is typically used as a rapid screening method to determine if particular ingredients are cytotoxic, whereas the ZK1200 [dermis and nondifferentiated epidermis] resembles the human cornea and is used for ocular testing."

Rosemarie Osborne, a senior scientist at Procter & Gamble's Human Safety Department, has experience with a number of three-dimensional alternatives. According to Osborne, an advantage of these analogs is the ability to apply test materials directly onto the tissue surface, without first diluting them in aqueous solution. "We were looking for a human cell-based system we could use for screening ocular irritants. We had a need to apply materials that are traditionally difficult to apply in vitro, such as granular laundry detergents. We wanted to test those in the same way in which people might encounter them, such as when these detergents accidentally get into a person's eye."

During testing, once an agent or chemical is applied to the skin analog, the response can be monitored by such markers as changes in cell morphology, expression of protein markers and inflammatory mediators, and cell membrane integrity. A specific effect is indicated by the presence of an endpoint marker. For example, inflammation is indicated by the presence of cytokine interleukin 1, a protein molecule that activates an inflammatory response in body tissues. The type of cell in the model being used, as well as the mechanism of action of the agent under investigation, help determine which of various endpoint markers are used. "The endpoint marker you choose is very much dependent on what kind of agent you apply topically," says Ponec. She goes on to explain that it is best to choose a battery of such markers because each agent has a specific mechanism of action that can be reflected by a variety of cell and tissue changes.

The potential to use some markers for the early detection of changes that occur more slowly during the inflammatory or repair process is a unique feature of many *in vitro* systems. Ponec, who is evaluating messenger RNA as an early marker, says, "If something is a strong irritating substance, you get an early yes or no answer which is fairly obvious. In daily life, you have a lot of mild irritants." Such irritants induce changes more slowly, with repeated use over a long period of time. For this reason, some investigators follow their cultures for days or weeks to observe subtle effects at the cellular or molecular levels, often before effects can be measured in *in vivo* counterparts.

Skin^2 is the only skin analog of its kind being marketed in the United States because there is little demand for these products so far. Biotechnology companies say this lack of demand is evidence of the fact that there is still not a major emphasis on developing and applying *in vitro* methods for toxicology testing. Still, Rheins remains hopeful: "Advanced Tissue Sciences continues to make strong efforts and investments in laboratory toxicology kits. The in vitro market, albeit small at present, will continue to grow with additional recognition and approval from the global regulators. We are also looking at ways to bring costs down while increasing availability across all sectors of the industry, including government and research."

The Eyes Have It

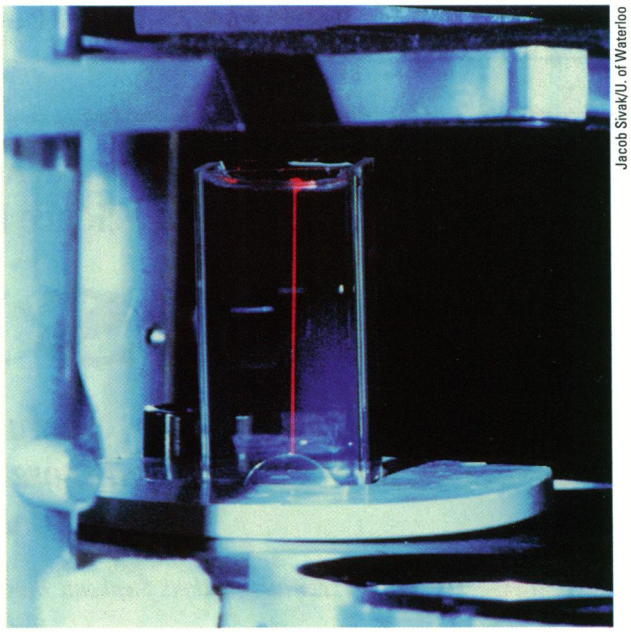
Among the scientific and moral concerns that drive the development of *in vitro* analogs, perhaps one of the strongest is opposition to the Draize eye test, which evokes strong sentiments among opponents to traditional animal testing. Jacob Sivak, at the University of Waterloo in Ontario, has developed an *in vitro* method for scanning the optical quality of cultured lenses. Sivak harvests lenses from the eyes of slaughterhouse cattle, using techniques that he developed while investigating damage to the eye from low concentrations of ultraviolet radiation. "The idea was to be able to take the lens out of the eye and keep it alive for long periods of time, then use it to test for possible causes of cataracts," he explains.

Lens cells are highly sensitive and readily reflect damage from very low doses of ultraviolet radiation. Anatomically, they consist of only epithelial cells, the same type of cell found on the outer surface of the cornea and on the surface of the body. All arise from the same embryological source and, as such, provide the scientific basis for using skin analogs to assess eye irritation.

Sivak uses the refractive ability of the lens as an endpoint when monitoring lens function *in vitro*. The lens is treated with a chemical and placed in a lens culture chamber which he describes as "similar to an artificial eye." A laserbeam is vertically aligned from below the chamber and passes through the lens, which refracts the light. This change in direction is filmed by a television camera and fed into a computer, which calculates the angle of refraction.

One of Sivak's greatest problems has been how to measure toxicity without damaging the lens. "Basically, we've developed a technique where we can dip the lens in a [toxic] solution for short periods of time," Sivak says. This allows him to test a range of toxicities, from highly toxic to "situations where the material is less toxic in very low concentrations," he says.

Sivak has tested a number of chemicals, including alcohols, surfactants, ketones, and acetates. He has also worked with companies such as Apotex Inc. in Toronto, to measure toxicity of various chemicals. "What we needed to do was assess the toxicity of some of the components of a vehicle [a substance used to deliver a drug] we were developing," explained William Jacobson, director of innovative drug development at Apotex. "The work was useful and helpful, and we used the results in our continuing formulation development," he said.



Jacob Sivak/U. of Waterloo

Wide angle lens? Effects of toxic substances can be assessed by directing a laserbeam through a cow lens and measuring the angle of refraction.

The ability to measure recovery from damage is a key issue in toxicity testing. "If the chemical does damage the eye, for example," says Sivak, "but the damage can be repaired, it is less of a risk than if the damage is permanent." Many early *in vitro* alternatives did not measure recovery. Sivak, who was recently awarded a grant from the Canadian government to specifically look at lens recovery, says that the intact lens retains its ability to repair damage, but damage and recovery do not always correlate. The degree of recovery varies: the lens with the greatest damage from a chemical may also show the greatest degree of recovery. To measure recovery in Skin^2 systems, the chemical is washed from the epithelial surface, which is then observed for several days to see if it returns to its normal physiological state, according to Rheins.

A World of Possibilities

Despite the currently small American market for skin analogs and other *in vitro* technologies, the pace of their development has picked up in recent years, due in part to the projections of the European Commission. The EC has targeted 1998 as the year that they will expect cosmetics companies to use *in vitro* alternatives whenever they are available and the methods are scientifically sound. "That has global ramifications. All these companies work in a global marketplace; they're not going to make different products to meet different *in vitro* testing needs," says Rheins.

William Stokes, associate director for animal and alternative resources in the Environmental Toxicology Program at the NIEHS, says that scientific validity is a key consideration in the directive. Stokes points out that the basic science involved in many of these *in vitro* systems may point the way to one of their most valuable roles, providing information about the mechanism of action of a particular toxic response. This in turn may allow regulators and investigators to better determine both the qualitative and quantitative relevance of the *in vivo* method.

Encouraging news about this kind of application grew out of a 1993 international workshop sponsored by the United States Interagency Regulatory Alternatives Group (IRAG). As a result of that meeting, "the U.S. IRAG has recently submitted a proposed testing scheme for acute ocular irritance," explained Stokes. "The proposal, which allows for the consideration of in vitro test data, was submitted to the Organization for Economic Cooperation and Development."

In this proposal, *in vitro* methods do not replace *in vivo* models, but data accrued using alternatives are considered in determining what type of animal testing should be done. Stokes describes it as a weight-of-evidence approach to toxicity, testing that considers all available information about a chemical including physical characteristics and chemical structure–activity data. This approach allows for scientifically based flexibility in the *in vivo* testing rather than the standardized approach often used.

As newer alternatives become available, they will continue to enhance toxicity testing while addressing the moral and ethical concerns of using live animal models. In defining the mechanism of action for chemicals, analogs continue to capitalize on what some believe is their greatest asset.

Mary Weideman

Suggested Reading

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